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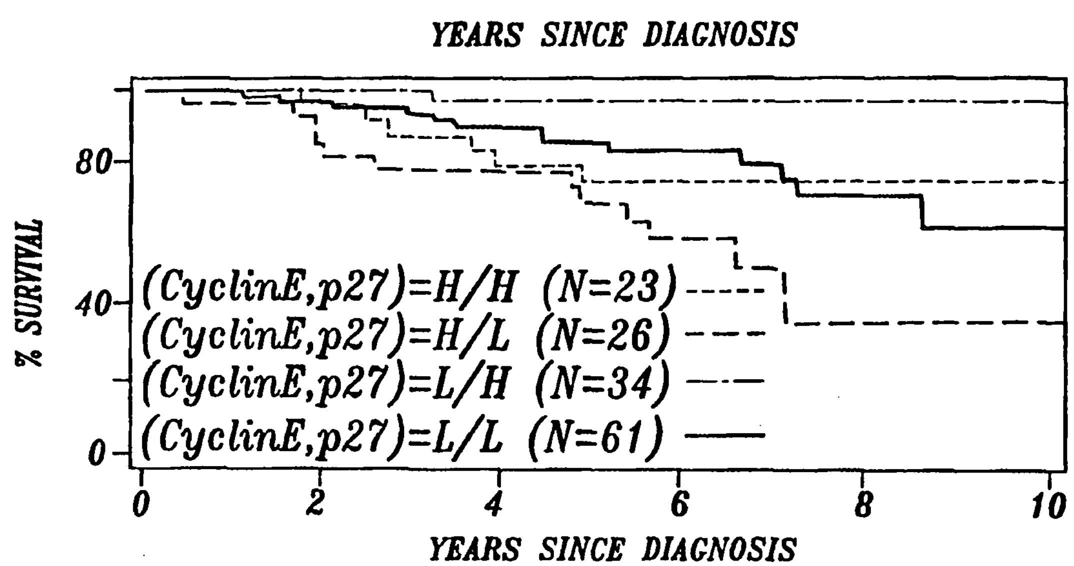

(57) Abstract

The subject invention provides methods for determining the prognosis for cancer patients and for staging cancer by analyzing tumor samples to determine the levels of both cyclin E and p27–kipl.

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PROGNOSIS OF CANCER PATIENTS BY DETERMINING EXPRESSION OF CELL CYCLE REGULATORS P27 AND CYCLIN E

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Field of the Invention

This invention provides methods for prognosis and staging in cancer patients involving the measurement in biological samples from cancer patients of the levels of expression of both the cell cycle regulators cyclin E and p27.

Background of the Invention

Mutations in genes that regulate the cell cycle are the most common genetic changes found in tumor cells (Clurman and Roberts, (1995)). Several studies have focused on the efforts to detect mutations associated with the cell cycle genes that encode cyclin E, a G1 cyclin, and p27-kip1 (hereinafter, "p27"), a CDK inhibitor. (Pietenpol, J., *et al.* (1995); Bhatia , K., *et al.* (1995); Ponce-Casteneda, M., *et al.* (1995); Konstantin, S., *et al.* (1996); U.S. Patent No. 5,543,291 (1996)). Various studies have suggested that the proteins encoded by these genes contribute to tumor progression (Leach, F., *et al.* (1993); Keyomarsi, K., *et al.* (1994); Keyomarsi and Pardee (1993); Said and Medina (1995); Fero, M.L., *et al.* (1996); Kiyokawa, H., *et al.* (1996); Nakayama, K., *et al.* (1996)).

Cyclin E, a regulator of the G1 to S-phase transition in mammalian cells, has been implicated in numerous types of human cancer (Koff *et al.* (1991); Lew *et al.* (1991)). Two isoforms of cyclin E, having sizes of 50 and 55 kilodaltons, have been

identified in cycling cells (Ohtsubo and Roberts (1993)). Cyclin E is a nuclear protein that attains its maximal level at the entrance to S phase, and then is degraded as S phase progresses (Gong *et al.* (1994); Ohtsubo *et al.* (1995)). Thus, the amount of cyclin E present at this critical stage in the cell cycle would appear to be a factor in a cell's ability to traverse S phase and to subsequently divide. Not unexpectedly, constitutive expression of cyclin E shortens the G1 phase of the cell cycle and promotes an increased rate of cell division, although the cells constitutively expressing cyclin E lack many of the characteristics of tumorigenic cells (Ohtsubo and Roberts (1993)). This latter finding suggests that overexpression of cyclin E alone is not sufficient to cause cancer.

Cyclin E, and other cyclins as well, manifests its control of the cell cycle by associating in the cell nucleus with other proteins called "cell division kinases" (CDKs) (U.S. Patent No. 5,549,755 (1995)). Association of CDKs with cyclins to form cyclin/CDK complexes results in the activation of the previously dormant kinase activity. Thus, the cyclins often are described as being the regulatory subunits of the CDKs. During the late G1 and early S phases of the cell cycle, cyclin E binds and activates at least two different kinases that belong to the "CDK2" family. Targets for phosphorylation of cyclin E-activated kinase include, for example, cyclin E itself, and histone H1. Levels of the cyclin E/CDK2 polypeptide complexes normally are cell cycle-regulated, and peak in abundance in late G1 phase of the cell cycle in accordance with the peak levels of cyclin E itself.

In recent years it has emerged that cyclin E is aberrantly expressed in a variety of human tumors (Leach *et al.* (1993) (cyclin E genes amplified in 2 of 47 colorectal carcinomas); Keyomarsi *et al.* (1994) (quantitative and qualitative alterations in cyclin E protein production in human breast cancer and leukemia; Garcia-Foncillas, J., *et al.*, (1996) (overexpression of cyclin E associated with decreased survival in nonmetastatic esophageal tumors); Gong *et al.* (1994) (cyclin E expressed in the wrong phase of the cell cycle in ductal breast carcinoma and colon carcinoma cells); Dutta *et al.* (1995) (cyclin E expression is deregulated in breast tumor samples); Said and Medina (1995) (elevated cyclin E expression is correlated with mouse mammary tumor progression); Nielsen, N.H., *et al.* (1996) (high levels of cyclin E in human breast cancer samples correlated with increased risk of death and relapse).

Like the cyclins that stimulate cell division, inhibitors of the cell cycle also play an important role in regulating progression through the cell cycle. The p27

protein belongs to this class of cell cycle regulators that, in contrast to the cyclins, inhibit cell division. The class of inhibitors to which p27 belongs acts by inhibiting the activity of the cyclin-dependent kinases, thus are known as the "kip" proteins. Normally, p27 is present in high levels in quiescent cells, and declines in proliferating cells in response to mitogenic signals such as growth factors and cytokines (Firpo, E. *et al.* (1994); Nourse, J., *et al.* (1994); Coats, S., *et al.* (1996)).

p27-kip1 specifically inhibits the kinase activity of the cyclin E/CDK2 complex by binding with cyclin E (Ponce-Castenada *et al.* (1995); Coats *et al.* (1996)). Coats *et al.* demonstrated that enforced expression of p27 arrested the cell cycle in G1, and that conversely, decreasing the level of p27 with antisense oligonucleotide inhibition resulted in a shortened G1 phase, and an increased rate of cell division. The down regulation of p27 in response to mitogenic signals has been proposed to be a critical step in mediating the response of normal cells to mitogenic stimuli (Coats *et al.* (1996)).

Because p27 slows cell proliferation by inhibiting CDKs, several groups have raised the possibility that p27 alterations could be involved in tumorigenesis. Not only is p27 involved in regulating the cell cycle, but also it has been mapped to the human chromosome arm 12p, which is a site of frequent deletions and rearrangements in a number of human cancers, including germ cell tumors, ovarian teratoma, leukemia, peritoneal mesothelioma, and malignant ovarian neoplasms (Pietenpol *et al.*, (1995); Ponce-Castenada *et al.* (1995)). This circumstance suggested the hypothesis that the non-deleted copy of p27 remaining in those tumor cells may have undergone further mutations such that the cells were left without a normal copy of p27. In this model, tumor progression would involve a process wherein one copy of p27 is first deleted, and the remaining copy is subsequently mutated.

Because the p27 gene thus seemed to be a likely "target" for mutation in tumor cells, Pietenpol *et al.* (1995) analyzed the p27 gene in bone marrow samples from 45 leukemia patients. They found a high proportion of hemizygosity for p27, but the remaining copies of the gene appeared normal. To explain their results, they proposed that the observed hemizygosity for p27 might result in reduced levels of p27 and a corresponding reduction in the level of CDK-inhibitory activity, thus resulting in uninhibited cell growth. Similarly, Ponce-Castenada *et al.* (1995) found no detectable cancer-specific deletions or point mutations in the p27 gene in a study of 147 human primary solid tumors, including bladder carcinomas, prostatic

carcinomas, pancreatic adenocarcinomas, breast carcinomas, lung carcinomas, germ cell tumors, melanomas, and soft tissue sarcomas. This group proposed that although abnormal p27 genes were uncommon in tumor cells, it remained possible that p27 expression was subject to posttranslational modifications or altered patterns that could upset the stoichiometric balance between p27 and cyclin-CDC complexes, thus resulting in unregulated cell growth.

Prognostic indicators for cancer are desirable because they provide physicians with a basis for determining the best treatment for individual patients. For most types of cancer, the prognostic indicators generally relied upon include tumor size, histopathological classification, and the results of lymph node biopsies. Based on these and other cancer-specific criteria, cancers typically are classified into various "stages," generally designated by the roman numerals I, II, III, or IV. In many cases, other indicators have been established whose prognostic value is associated with only one or a limited number of tumor types (McGuire and Clark (1992)). Nonetheless, prognostication of cancer remains imperfect, and many patients continue to be either undertreated or overtreated. Improved prognostic methods can assist physicians in better determining which patients require aggressive treatment, and which ones will thrive with only the minimal degree of therapy, thus improving the average survival of all cancer patients.

Summary of the Invention

It has now been discovered that high levels of cyclin E and low levels of p27 are strongly predictive of increased mortality in cancer patients, both before and after adjustment for other clinical and pathological characteristics. Most dramatically, it has now been demonstrated that when cyclin E and p27 indices are combined, the pattern of low p27 and high cyclin E expression is associated with a multi-fold increased relative risk of mortality.

Accordingly, this invention provides methods for determining the prognostic outcome of cancer, and for assigning tumors to various stages of tumor progression. These methods involve obtaining from the patient a biological sample that either contains cancerous tissue, such as a tumor biopsy, or a sample such as blood or urine that contains materials or molecules derived from tumor tissues that have become necrotic or that have otherwise released their contents. The levels of expression of cyclin E and p27 are then determined for these tumor samples. By comparison with a set of standards, the observed levels of expression of cyclin E and p27 in the patient sample are classified as "high," "intermediate," or "low". When high levels of

cyclin E and low levels of p27 are observed, this is indicative of advanced stages in tumor progression, and the prognosis for such patients is poor with respect to relapse or death from cancer. A low level of cyclin E and a high level of p27 expression indicates a good prognosis, and indicates a low grade of tumor corresponding to the lower stages in tumor progression. Intermediate levels of expression of the two markers will correspond to intermediate stages in progression of the disease.

Brief Description of the Drawings

FIGURES 1A-1F illustrate the associations of cyclin E and p27 expression in breast tumor samples and survival of the patients from whom the samples were obtained (Kaplan-Maier plots). The FIGURES show survival in either the total group of women (FIGURES 1A-1C), or in the subset consisting of node-negative women (FIGURES 1D-1F). Each plot shows either the correlation between survival and levels of cyclin E alone (FIGURES 1A and 1D), levels of p27 alone (FIGURES 1B and 1E), or cyclin E and p27 combined (FIGURES 1C and 1F).

Detailed Description of the Preferred Embodiment

This invention provides methods for determining the prognostic outcome of cancer and for staging tumors. It has been demonstrated that measuring the levels of expression of cyclin E and biological samples from cancer patients provide a prognostic index having greater predictive value than measurements of either cyclin E or p27 alone. Thus, measurement of these two indicators in the same biological sample provides prognostic information valuable for determining the best therapeutic protocol for treating cancer patients, a group that includes patients suffering from various types of cancer, including sarcoma, melanoma, leukemia, myeloma, and carcinoma, including breast carcinoma, prostate carcinoma, colorectal carcinoma, stomach carcinoma, esophageal carcinoma, bladder carcinoma, cervical carcinoma, lung carcinoma, as well as other cancers.

The subject invention thus provides a method for determining the prognostic outcome of cancer that involves measuring the levels of expression of cyclin E and p27 in a tumor sample and comparing the levels observed in the sample with the levels of expression in a set of standards. For purposes of these descriptions, it should be understood that a "tumor sample" includes samples derived from any patient suffering from cancer, including those forms of cancer, e.g., leukemia, that are not typically associated with the formation of solid tumor masses. If the patient from whom the tumor sample is taken has, as compared with the standards, a high level of cyclin E expression, and a low or undetectable level of p27 expression, this

circumstance indicates that a poor prognostic outcome can be expected for that patient. Cancer patients having a "poor prognostic outcome" are those who are significantly more likely than other patients having the same type of cancer to have a relapse or to die from the cancer at the end of any designated test period. "Significantly more likely" in this context refers to statistical significance, where the relative risk of death (RR) is calculated according to conventional statistical methods. Moreover, the relative levels of cyclin E and p27 can be used to stage cancers as an adjunct or as an alternative to conventional methods for cancer staging.

The values for "low," "intermediate," or "high" levels of expression are determined by comparison to reproducible standards in which low or high levels of expression have been demonstrated to be present. The tissues used to establish these standards can be derived from the normal tissue found adjacent to tumor tissue in biopsy samples, from normal tissue taken from the same tissue type in non-cancer patients, from normal tissues of other types, or from cultured cells that are determined empirically to express low, intermediate, or high levels of these two prognostic markers. The levels measured in these control tissues thus establish a range from "low" to "high," and once these are established, the levels of these markers expressed in tumor tissues can be compared with levels present in the standards, and thus be assigned a value of "low," "intermediate," or "high."

The term "tumor sample" may include any tissue or body fluid from a cancer patient and includes either malignant cells or materials or molecules derived from malignant cells. Hence, a "tumor sample" includes a body fluid into which the contents of tumor cells have been released (e.g., blood, or could be urine), and which may contain metabolic degradation products derived from the tumor. Thus, as used herein, "tumor sample" refers not only to tumor biopsy samples, but also to samples of blood, saliva, urine, skin scrapings, or any other tissue derived from the patient's body. Tumor samples can be analyzed by measuring intact mRNA or protein expressed by the cyclin E or p27 genes, including aberrant forms of these proteins. and also can be analyzed by measuring various breakdown products of these molecules that may be present, for example, in blood or urine.

By applying the subject method, cancer patients can now be subdivided into groups having either significantly elevated or significantly reduced risk of mortality based on their levels of expression of cyclin E and p27. Thus, these indices are useful in determining which patients suffering from cancer will benefit from more aggressive therapy.

Assays for determining levels of cyclin E and p27 in tumor samples include methods for quantifying mRNA specific for these proteins (such as, e.g., Northern blots), methods for direct detection of the proteins (e.g., western blots or immunocytohistological methods), or assays based on detecting functional activities of free cyclin E (e.g., phosphorylase kinase activation) or complex-associated cyclin E (ability to phosphorylate histone H1). Similarly, functional assays for p27 are based on this protein's ability to inhibit cyclin E/CDK kinase activity. Functional assays for both cyclin E and p27 are known in the art (e.g., Koff *et al.* (1991); Polyak, K., *et al.* (1994a); Polyak, K., *et al.* (1994b)).

The subject invention includes assays for: a) detecting the relative or absolute levels and activities of cyclin E and p27 in tumor samples including nonsynchronized cell populations (e.g., in tumor biopsy specimens or body fluids of cancer patients); and b) assays for determining the levels and activities of cyclin E and p27 in various types of biological samples (i.e., solid tissue samples, primary cultures of tumor cells, blood, urine, saliva, serum, plasma, mucus secretions, CNS fluid, cell extracts, and the like). The levels and activities of cyclin E and p27 expressed in these tumor samples, taken together, provide an improved method of determining the stage and predicting the outcome of cancer in individual patients. Analysis of this pair of markers can be used either as an alternative to present methods of diagnosis, prognosis, and staging, or as an adjunct to extant risk factor analysis.

Simultaneous analysis of p27 and cyclin E levels can be used for determining prognosis, i.e., predicting patient survivability and time to recurrence of tumor, for forming a basis for determining if aggressive anti-cancer therapies are appropriate (e.g., chemotherapy or irradiation therapy), for monitoring the effectiveness of ongoing therapy (e.g., by analyzing biopsies taken at various times during treatment), or for staging tumors.

Malignant condition often are classified into stages ranging from Stage I to IV. Staging is useful to facilitate planning the most appropriate course of therapy for each patient and for predicting the likely outcome of the disease. Each cancerous condition has its own staging classification, as each cancer is different, but the stages are broadly defined as follows. Stage I is defined usually as cases wherein the cancer is still confined to the organ in which it originated. Such tumors are considered to be operable, and the prognosis is typically favorable. Stage II cancers usually involve some surrounding tissue, while Stage III cancers have invaded the local lymph nodes.

In Stage IV patients, the cancer has metastasized into areas of the body distant from the original site, and organs besides the one in which the cancer originated have become involved. In general, the higher the staging number, the more serious the cancer, and the poorer the prognosis. However, prognostication based on conventional histological staging is imperfect, and physicians usually take other factors into account as well in making decisions regarding treatment.

The subject invention offers methods for staging tumors that can improve the physician's ability to predict the course of cancer in individual patients. To establish a correlation between tumor stages and expression of p27 and cyclin E, conventionally staged tumors from various types of cancer are analyzed for cyclin E and p27 levels, and thereafter, measurement of these levels can serve as a surrogate, or as an adjunct, to the conventional staging analyses.

For staging, a tumor sample is obtained from a cancer patient, and the levels of expression of cyclin E and p27 in the tumor sample are assayed. Thereafter, the levels measured in the tumor sample are compared with the levels present in a set of standards whose content of cyclin E and p27 have been established to correspond to the levels found in Stages I, II, III, or IV for the same type of cancer from which the tumor sample is derived. Thus, the cancer from which the tumor sample was derived can be assigned a classification based on the correspondence of the levels measured in the sample with the levels present in the standards.

In an exemplary application of the subject invention, the expression of cyclin E and p27 was characterized in breast tumors from a group of 278 young women. Also assayed in these samples were a number of other indicators that had been implicated previously as independent prognosticators of breast cancer. The results, described below in Example 1, indicated that when the cyclin E and p27 indices were combined, this combination provided a prognostic indicator far better than any one of the indicators taken alone. The superiority of this combined index is apparent from the plots shown in Figures 1A-1F.

EXAMPLES

Example 1: Distribution of Cyclin E and p27 in Normal and Carcinoma Tissues

Because breast cancer is now recognized to disseminate early during its course, prognostic indicators are of particular importance in this disease. To date, efforts to predict the course of the disease have relied largely on the presence of metastases in axillary lymph nodes, tumor size, and grade, and tumor proliferation markers. Adjuvant therapy after surgical removal of the tumor has become the

standard of care for the majority of women with axillary node involvement, however, the efficacy of adjuvant therapy in node negative patients is much less certain (McGuire and Clark (1992); Conference, N.C. (1991)). Tumor size has been used to stratify patients into prognostic groups, however, almost 50% of node negative women fall into a category of intermediate tumor size with no clear indication for treatment choice (McGuire and Clark (1992)).

Prognosis of breast cancer is important, especially in node-negative patients, because while following excision two-thirds of these women will do well without further adjuvant therapy, the remaining third will experience relapse (Dutta *et al.* (1995)). Because of the deleterious side effects, aggressive treatment of all node-negative women is not considered worth the risks and side-effects associated with such treatment. However, node-negative women having a poor prognosis could be identified at the time of initial diagnosis, these women could receive the aggressive therapy that would increase their survival.

The measurement of cyclin E levels has recently emerged as being a good prognosticator for breast cancer, and the overexpression of cyclin E protein was shown to be associated with a two-fold greater risk of death (Keyomarsi *et al.* (1994) (altered expression of cyclin E correlates with increased breast tumor stage and grade); Said and Medina (1995) (increased cyclin E expression in tumorigenic cell lines); Nielsen *et al.* (1996) (increased cyclin E expression associated with decreased survival of breast cancer patients); Dutta *et al.* (1995) (elevated cyclin E expression associated with breast cancers having a high proliferative index). Moreover, abnormal isoforms of cyclin E have been observed in breast tumors (Keyomarsi, K., *et al.* (1994); Said and Medina (1995)).

To provide prognostic assays for breast cancer and other forms of cancer, experiments were conducted as described below to determine the relative distribution of cyclin E, p27, and c-erbB-2, in human tonsil tissue, benign breast epithelium, and invasive ductal carcinoma.

<u>Patient population</u>

Available for this study were paraffin-embedded primary breast tumor tissue samples, obtained prior to any adjuvant treatment, from a cohort of 1292 women, aged 20 to 44, who were identified through the Cancer Surveillance System (CSS) of western Washington and who were interviewed as part of a ongoing study at the Fred Hutchinson Cancer Research Center. These women were diagnosed between 1983 and 1992. A total of 278 ductal carcinoma samples from this cohort were analyzed.

Forty-eight percent of the women from whom these samples were derived were node positive. Information concerning diagnosis date, tumor size, clinical stage, and lymph node status, and deaths was obtained from the CSS. Subjects were followed until the earliest of: their date of death, the date last known to be alive, or the end of the follow-up period. Observations were censored at either the date of last known follow-up or the end date of the follow-up period if death had not occurred.

Compared with women in the entire cohort, the subject of women tested for this study were more likely to have been diagnosed in the early years of the study (40% vs. 57%) and were more likely to have died (53% vs. 87%). A weighted Cox regression analysis accounted for differences in the probability of testing and allowed for valid relative risk evaluation (see statistical methods section). Other clinical characteristics (age at diagnosis, stage at diagnosis, lymph node status, and tumor size), varied no more than 7% between the entire cohort and the women tested for this study.

Tissue evaluation and characterization of antibodies

Each of the tumors analyzed for this study was assigned a histologic grade from I (low) to III (high) according to the Bloom and Richardson grading scheme for invasive ductal carcinoma.

Antibodies used for this study included previously characterized affinity purified polyclonal anti-cyclin E (Ohtsubo, M., *et al.* (1995)), anti-cyclin A (Roberts' lab), and anti-p27 (Nourse, J., *et al.* (1994)); rabbit polyclonal anti-c-erbB-2 (Dako, Denmark); anti-p53 clone 1801 (Oncogene Science, Uniondale NY); anti-Ki-67 clone MIB-1 (Immunotech, Westbrook ME). Experimental validation of immunostaining with anti-cyclin E antibody was done by constructing cell lines that overexpress cyclin E from a transfected gene. To provide standards for assessing the levels of cyclin E in test samples, cyclin E immunostaining was compared with western blotting and a direct correspondence between the amount of cyclin E present and the intensity of cyclin E immunostaining was established (Ohtsubo, M., and Roberts, J. (1993)). Tissues from p27 null mice (Fero, M.L., *et al.* (1996)) provided a negative control for the p27 antibody; in the absence of p27, no detectable immunostaining was observed with the anti-p27 antibody. Standards for "low," "intermediate," and "high" levels of p27 immunostaining were established using, respectively, 1) p27 cells derived from p27 null mice (negative), 2) proliferating rat fibroblasts (intermediate) and 3) serum starved quiescent cells (high). By performing western blots on these three types of cells using anti-p27, it was established that a

direct correspondence existed between the amount of p27 in the cells and the intensity of p27 immunostaining.

Immunohistochemical Studies

All scoring and interpretations of immunocytochemical results were made by a single pathologist (PLP), who had no knowledge of the clinical outcome, other clinical variables, or presence or absence of tumor markers in each example. Paraffin blocks for testing were selected for presence of representative tumor and, when available, presence of adjacent benign epithelium in the same block. Immunostaining was done using a modification of the standard immunoperoxidase technique, which included microwave treatment of the tissue sections in the presence of citrate buffer (Gerdes, J., *et al.* (1992)).

Four high power fields (400X magnification) from a single representative tissue section, chosen to reflect the area of highest cyclin E or p27 intensity, were scored and each tumor section was assigned a single composite value from 0 (negative) to 6 (highest intensity) that reflected both staining intensity and the percentage of tumor cells positive. Categories of "high" and "low" cyclin E and p27 immunostaining were determined by comparison of assays in benign breast epithelium. For cyclin E, "low" intensity included all values of 0-3 (98% of benign epithelial samples) and "high" included values from Ü4-6 (2% of benign epithelial samples). Levels of staining for p27 in benign epithelium ranged widely from values of 2-6, and staining above levels 0-1 was almost always present (77%). "Low," "intermediate," and "high" categories of p27 expression were assigned as 0-1, 2-3, and 4-6 respectively. For regression analysis, intermediate and high categories were combined and compared to low. Five percent of the cyclin E and p27 assays were re-scored for a reliability assessment, blinded to the first reading. In these re-assessments, the categories of staining intensity never varied by more than 1 integer and there were no instances where the difference in the first and second review resulted in changing the case from one summary category ("high" vs. "low") to another.

Statistical Methods

Associations between the cell cycle proteins and covariates were calculated using contingency table methods and tested for significance using Pearson's chi-square test. Survival curves were calculated using the Kaplan-Meier method. These curves are used to graphically display subset comparisons and are not intended to characterize absolute mortality rates within the cohort particularly since the sampled

subset has an over representation of deceased women. Univariate and multivariate relative risks were computed using Cox proportional hazards regression where observations for this subset of women have been weighted proportional to the inverse of their sampling probability with respect to the larger study. Such weighting allows valid estimation of relative risks in non-standard sampling schemes such as survey data or where covariates are missing (Lin and Ying (1993); Binder, D. (1992)). Sampling weights were used that depended on vital status and year of diagnosis as discussed in the patient population section above. All calculations were performed using S+ version 3.3 (StatSci, WA, USA).

Results

Sections of human tonsil tissue, benign breast epithelium, and invasive ductal carcinoma were subjected to immunohistochemical analyses as described above. When human tonsil was immunostained with anti-cyclin E, it was revealed that cyclin E was present in the nuclei of scattered cells of the proliferative germinal center, and was absent from the quiescent mantle zone cells. p27 exhibited the inverse pattern in human tonsil, in that it was absent from the germinal center, and instead was observed predominantly in the quiescent cells of the mantle zone. Different staining patterns for cyclin E and p27 also were observed in benign breast sections, in which cyclin E was absent, and p27 was present in the nuclei of epithelial cells. Breast cancer cells exhibited high levels of cyclin E in a poorly differentiated tumor and, conversely, exhibited high levels of p27 in a well differentiated tumor.

The levels of expression of cyclin E and p27 were compared in this same group of samples to other tumor characteristics and risk factors (Table 1) and to mortality after a median follow-up of 5.2 years. The relative risks (RR) (both univariate and multivariate) of dying and 95% confidence intervals (CI) were estimated using a weighted Cox proportional hazard model, among 237 women for whom information concerning stage, age at diagnosis, tumor size, lymph node status, histologic grade, and assays of cyclin E, p27, Ki-67 proliferation index, p53, and c-erbB-2 were available. In univariate models, positive lymph nodes, large tumor size, intermediate, and high histologic grade, presence of c-erbB-2, high levels of cyclin E, and low or absent p27, were associated with increased risk of death (Table 1). However, after adjusting for all other factors, only lymph node status, presence of c-erbB-2, high cyclin E levels, and low p27 levels, were associated with decreased survival (Table 1).

Table 1: Univariate and multivariate analyses comparing overall survival to prognostic factors in 246 breast cancer patients.

Overall Survival

Prognostic factor	Univariate (P)	RR (Univariate)	Multivariate (P)	RR (Multi-variate)
positive lymph nodes	<0.001	4.5 (2.8-7.0)	<0.001	4.5 (2.3-8.8)
tumor size (cm)				
2-4+	<0.001	2.8 (1.6-4.9)	0.09	1.9 (0.9-4.0)
5 or >	<0.001	7.0 (3.1-15.9)	0.08	2.9 (0.9-9.7)
histological grade				
intermediate	0.007	3.1 (1.4-6.9)	0.37	1.6 (0.6-4.8)
high	<0.001	3.9 (1.7-8.6)	0.18	2.1 (0.7-6.4)
c-erbB-2	<0.001	2.4 (1.5-3.9)	0.04	2.0 (1.1-3.9)
p53	0.10	1.5 (0.9-2.5)	0.90	1.0 (0.4-2.2)
Ki-67 high	0.40	1.2 (0.8-2.0)	0.49	0.7 (0.3-1.7)
high cyclin E level	0.001	2.1 (1.3-3.3)	0.03	2.4 (1.1-5.2)
low p27 level	<0.001	2.9 (1.7-4.9)	0.01	2.7 (1.3-6.0)

The factors analyzed for this group of samples are listed in Table 1. The Cox regression model was used to evaluate the univariate and the multivariate predictive value of prognostic factors; results are shown as P (regression coefficient +/- SE). Relative risk was determined by the Cox regression model. Ninety-five percent confidence intervals are shown in parentheses. For Ki-67 analysis, positive tumor nuclei were scored in 4 fields (400X), which were selected to reflect areas of highest proliferation. For this analysis, "low" Ki-67 meant that 0-50% of the cells were positive for this marker, and "high" Ki-67 meant that 51-100% of cells in the observed fields were positive. No significant differences in the results were observed when the cut-off point between high and low values was set at either 25% or 50%. For p53 analysis, positive tumor nuclei in 4 fields were assessed, and the sample was considered positive if >10% of the tumor nuclei were positive for this marker. Membranous staining pattern of any intensity was considered a positive result for the c-erbB-2 marker.

When tumor and clinical characteristics were analyzed according to lymph node status in univariate regression models, large tumor size (RR 10.9, CI 1.4-83.1;

p=0.02), presence of c-erbB-2 (RR 3.4, CI 1.4-7.9; p=0.005), presence of p53 protein (RR 2.7, CI 1.2-6.5; p=0.02), high levels of cyclin E (RR 3.3, CI 1.5-7.4; p=0.003), and low or absent p27 (RR 4.1, CI 1.4-12.1; p=0.01), were associated with increased risk of dying in node negative women. In women with lymph node involvement, only the presence of c-erbB-2 (RR 2.4, CI 1.2-4.9; p=0.01), and low or absent p27 (RR 2.6, CI 1.3-5.4; p=0.007) were associated with decreased survival.

The Kaplan-Meier plots illustrated in FIGURES 1A-1F show the association of survival and expression of cyclin E and p27 in all women tested (FIGURES 1A-1C) and in node negative women (FIGURES 1D-1F). These plots show an increased mortality risk associated with high levels of cyclin E (RR 2.1, CI 1.3-3.3, p=0.001) (FIGURES 1A and 1D), and low levels of p27 expression (RR 3.9; CI 2.0-7.5; p=<0.001) (FIGURES 1B and 1E). Among FIGURES 1A-1D, the greatest difference in survival was found between women having virtually no p27 and women having the highest levels.

Stratification of survival based on 3 levels of p27 immunostaining was possible in the entire group of tested patients and reflected the increasing mortality associated with low to absent p27 (p<0.001) (FIGURE 1B; thus, these data provide a basis for tumor staging based on p27 levels). The association was also present in node negative women with low p27 (p=0.01) (FIGURE 1E) (the high and intermediate levels were included in the "high" category for analysis).

When cyclin E and p27 results were combined, stratification based on combinations of expression of the two proteins was possible in both groups of women (FIGURES 1C and 1F). Women with high cyclin E and low p27 expression experienced the highest mortality in the both groups (p<0.001). A striking stratification of mortality risk was identified when four different combinations of p27 and cyclin E proteins levels (i.e., both markers at high levels, both markers at low levels, cyclin E high when p27 is low and cyclin E low when p27 is high), were evaluated for their relative effect on survival (FIGURES 1C-1F). The combination of expression that corresponded most closely to normal breast tissue (high p27 and low cyclin E), was associated with the most optimal survival; the opposite pattern (low p27 and high cyclin E), was associated with the highest mortality (RR 8.6; CI 3.6-20.4; p<0.001).

Of the other tumor markers assessed in node negative women, increased S-phase fraction measured by flow cytometry is one of the more reliable indictors of tumor behavior. Although a strong overall correlation of cyclin E expression with

proliferation ($p<0.001$) was found, in 27% of the tumors analyzed the Ki-67 index and cyclin E expression levels were not concordant (in contrast to expression of cyclin A which invariably correlated with the proliferative status of the tumor). Compared with women whose tumors exhibited low tumor proliferation and low levels of cyclin E, women with discordant cyclin E expression and proliferation, i.e., low Ki-67 index and high cyclin E levels, showed a strong association with mortality (RR 3.3; CI 1.7-6.3; $p<0.001$).

These findings thus indicate that p27 and cyclin E are independent prognostic markers for cancer, and that measuring the levels of both indicators provides valuable prognostic information.

While the preferred embodiment of the invention has been illustrated and described, it will be appreciated that various changes can be made therein without departing from the spirit and scope of the invention.

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32. U.S. Patent No. 5,543,291

33. U.S. Patent No. 5,549,755

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A method for determining the prognostic outcome of cancer in cancer patients comprising the steps of:

(a) obtaining from the patient a tumor sample;

(b) measuring the levels of expression of cyclin E and p27 in the tumor sample;

(c) comparing the levels of expression of cyclin E and p27 in the tumor sample with the levels of cyclin E and p27 in a set of standards; and,

(d) determining that the patient has a poor prognostic outcome if relative to the standards the tumor sample expresses high levels of cyclin E, and low or undetectable levels of p27.

2. The method of Claim 1, wherein the cancer patient is selected from the group consisting of sarcoma, myeloma, leukemia, leukosis, melanoma, and carcinoma patients.

3. The method of Claim 2, wherein the cancer patient is a carcinoma patient.

4. The method of Claim 3, wherein the carcinoma patient is selected from a group consisting of breast carcinoma, prostate carcinoma, colorectal carcinoma, stomach carcinoma, bladder carcinoma, liver carcinoma, cervical carcinoma, and lung carcinoma patients.

5. The method of Claim 4, wherein the carcinoma patient is a breast carcinoma patient.

6. The method of Claim 4, wherein the carcinoma patient is a prostate carcinoma patient.

7. A method for classifying cancers into stages comprising the steps of:

(a) obtaining a tumor sample from a cancer patient;

(b) measuring the levels of expression of cyclin E and p27 in the tumor sample;

(c) comparing the levels of expression of cyclin E and p27 in the tumor sample with the levels of cyclin E and p27 in a set of standards whose levels of expression of cyclin E and p27 correspond to those found in Stages I, II, III, or IV of the cancer; and

(d) classifying the cancer into the Stage for which the levels of cyclin E and p27 in the standard for that stage corresponds to the levels in the tumor sample.

8. The method of Claim 1, wherein the tumor sample is selected from the group consisting of tumor biopsy, blood, urine, serum, plasma, saliva, mucous secretions, CNS fluid, cell extracts, or primary cultures of tumor cells.

9. The method of Claim 8, wherein the tumor sample is a biopsy sample.

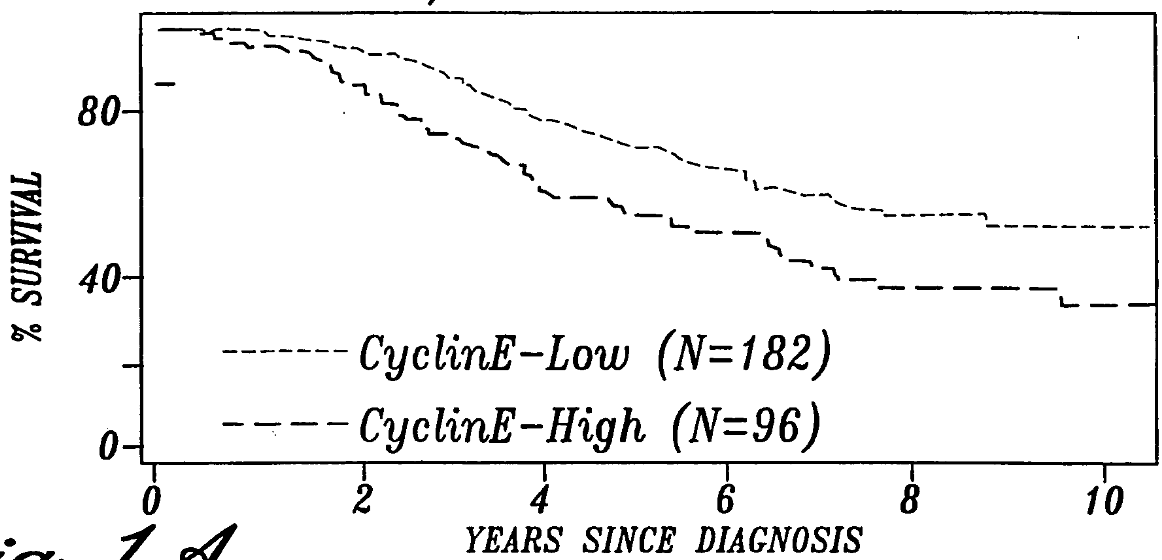

Fig. 1A.

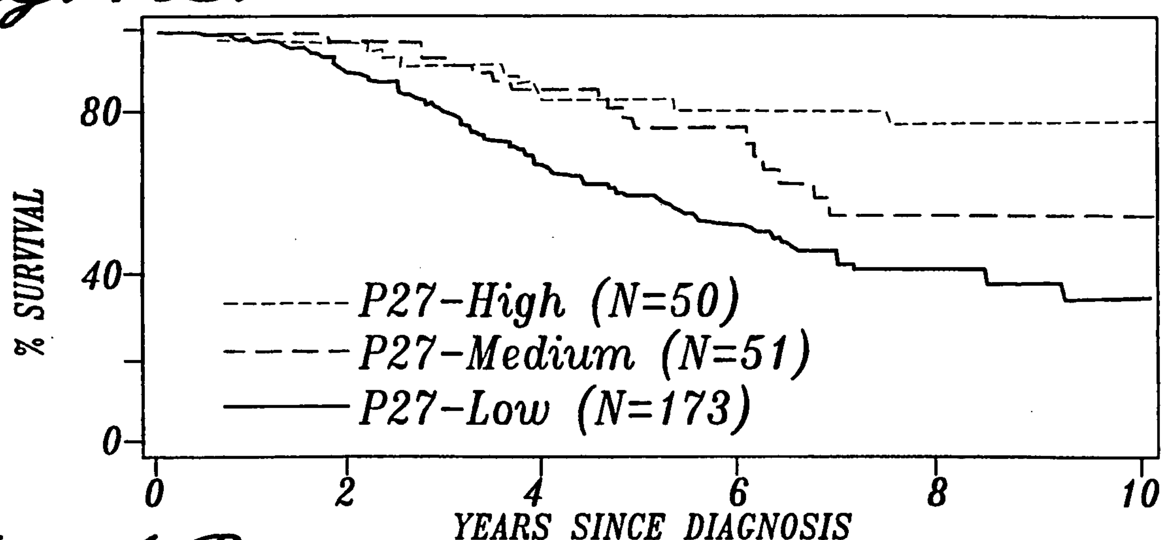

Fig. 1B.

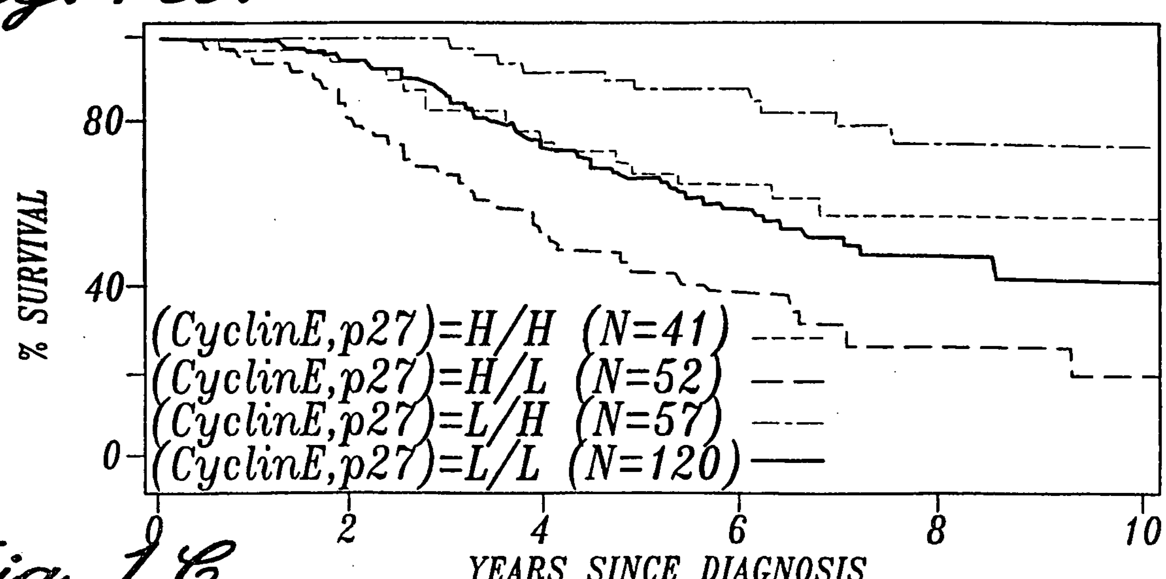

Fig. 1C.

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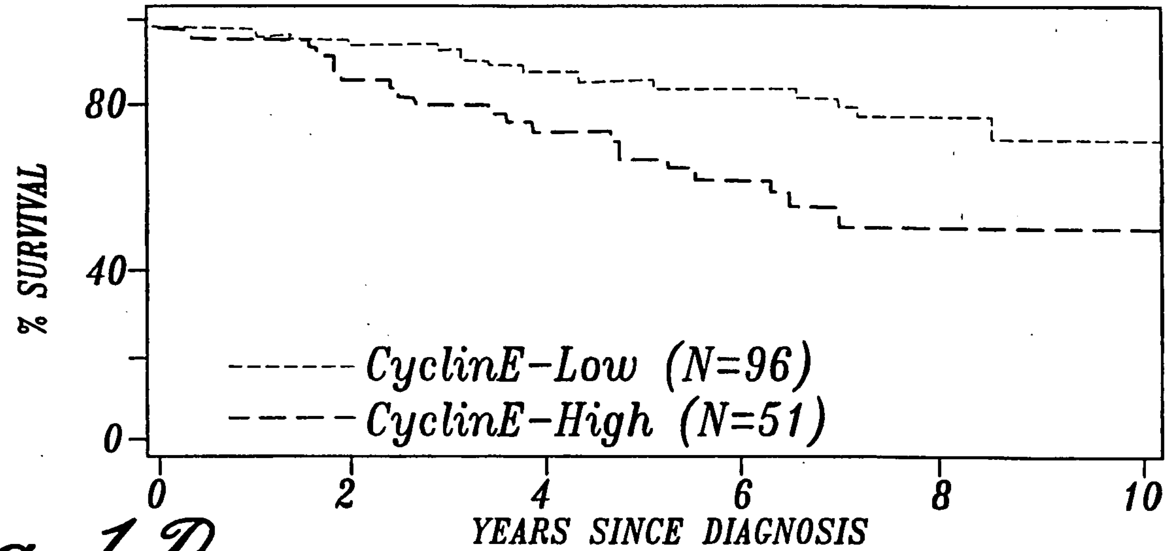

Fig. 1D.

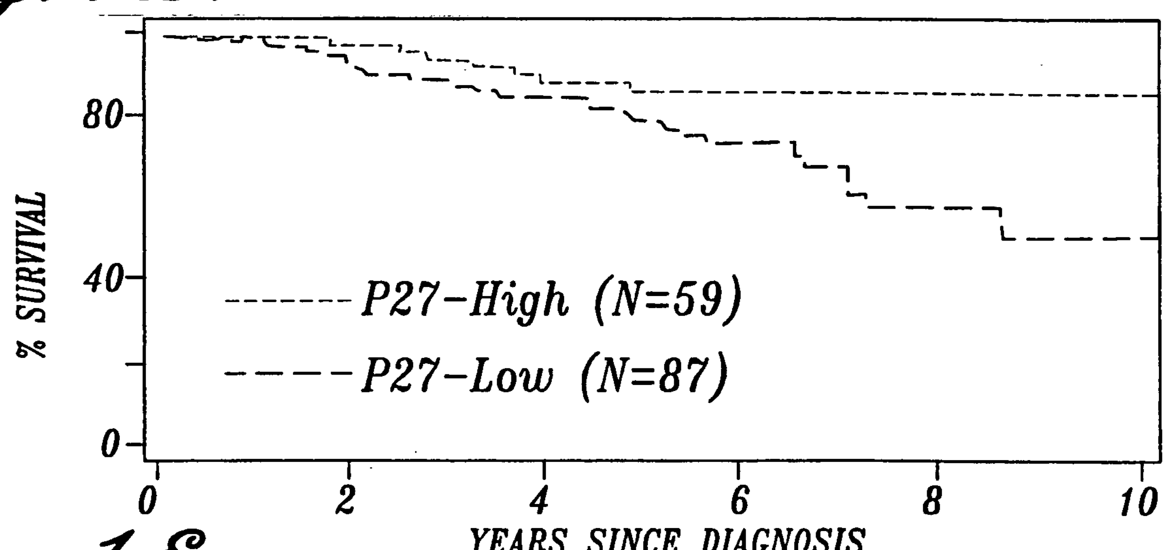

Fig. 1E.

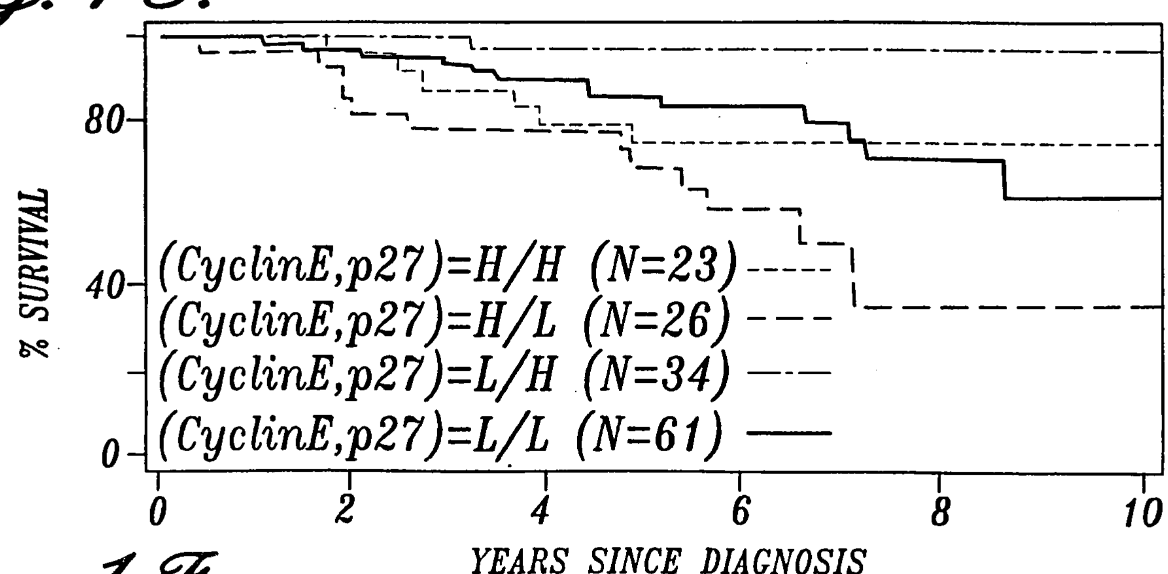

Fig. 1F.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/01922

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61B 19/00; GO1N 33/48, 574
US CL :128/898; 435/7.23; 436/63, 64, 813
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 128/898; 435/6, 7.23, 69.1, 69.2; 436/63, 64, 813; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,543,291 A (KEYOMARSI et al) 06 August 1996, entire document.	1-9
X,P ----- Y,P	US 5,688,655 A (MASSAGUE et al) 18 November 1997, entire document.	1-4, 7-9 ----- 5, 6
X,P	US 5,672,508 A (GYURIS et al) 30 September 1997, entire document.	1-9
X,E	US 5,733,920 A (MANSURI et al) 31 March 1998, entire document.	1-9

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 APRIL 1998	05 MAY 1998

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